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Concurrent decrease of vasopressin and protein kinase C α immunoreactivity during the light phase in the vole suprachiasmatic nucleus

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Abstract

Vasopressin (AVP) is a major neuropeptide in the suprachiasmatic nucleus, the mammalian hypothalamic circadian pacemaker. Protein kinase C α is a putatively coupled intracellular messenger. Mean numbers of AVP- and protein kinase C α -immunoreactive neurons were determined in the suprachiasmatic nucleus of common voles, entrained to a 12:12 h light-dark (LD) cycle, at the beginning of the light period (zeitgeber time zero) and 6 h later (zeitgeber time six). At zeitgeber time zero, mean numbers of AVP- and protein kinase C α -immunoreactive neurons were 2194 and 9897, respectively. Both numbers decreased significantly with about 40% at zeitgeber time six. This concurrent decrease was most pronounced in the dorsomedial aspect of the suprachiasmatic nucleus. These findings are consistent with the findings of a peak of AVP release in rats during the early light phase. © 1998 Published by Elsevier Science Ireland Ltd. All rights reserved

Keywords: Common vole; Suprachiasmatic nucleus; Vasopressin; Protein kinase C α ; Immunocytochemistry; Zeitgeber time

The neuropeptide vasopressin (AVP) is produced abundantly in the main mammalian circadian pacemaker, the suprachiasmatic nucleus (SCN). AVP-immunoreactive (AVP-ir) cells are predominantly localized in the dorsomedial zone of the SCN [20]. AVP-ir neurons form a complex and dense network, contacting AVP-immunopositive neurons as well as other neurons synaptically. The neuronal AVP circuit constitutes an important output system: AVP-labeled fibers arising from the SCN project to other hypothalamic regions [7], while considerable amounts of AVP are secreted in the third ventricle in a circadian rhythmic fashion [15]. There are good indications that AVP produced in the SCN is involved in the regulation or modulation of behavioral programs. The neuronal AVP-ir population in the SCN correlates with expression of circadian rhythmicity in common voles [6], and AVP-ir neurons in the mouse SCN have been implicated in relaying timing information of circadian wheel running activity [2]. Within

the SCN, AVP seems to play a role in circadian activity cycles [11], and it has been shown that AVP exerts excitatory effects on SCN neurons in vitro via the V1a receptors [8]. Activation of these receptors leads to membrane related phosphoinositol turnover and the subsequent activation of protein kinase C (PKC) [16]. PKCs are intracellular messengers, some of them dependent on calcium, and involved in a variety of processes regulating cellular excitability. Localization of the four Ca²⁺-dependent PKC-isoforms in the SCN has been established and they occur in the rat, mouse, rabbit and the diurnal Kusu rat, *Arvicanthis niloticus*, in different rates of expression [3,18]. PKC α is the isozyme that, immunocytochemically, is most abundant in the SCN of these species. PKC α is highly expressed in mouse AVP-ir SCN neurons and PKC α -ir SCN neurons are densely innervated by AVP-positive terminals [18]. The role of PKC α in the circadian pacemaker mechanism is currently unknown, but its activation could contribute to a wide variety of intracellular processes.

The common vole (*Microtus arvalis*) is characterized by rigid ultradian (2–4 h) rhythms in behavior [5], while the

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circadian component is less stable. The large interindividual variability in expression of circadian rhythmicity under constant conditions and the relationship with the number of AVP-ir cells in the SCN [6], allows the study of the circadian output system at clock level in more detail. The rat SCN shows a peak in content and subsequent release of AVP in the early hours of the light period, while there is a decrease of AVP-ir SCN neurons measured by optical densities [13,15,17]. If it is true that PKC α is the primary PKC isoform involved in AVP signal transduction, we expect a concurrent decrease in the total number of AVP and PKC α -ir cells in the SCN during the early morning in voles with behaviorally circadian rhythmicity. Therefore, the aim of this study is to investigate possible changes in AVP- and PKC α -ir cells in entrained voles, housed under light-dark (LD) conditions, and sacrificed at the immediate beginning of the light period (zeitgeber time 0, ZT 0) or 6 h later (ZT 6).

Fourteen adult male voles (3 months old), showing strong expression of circadian rhythmicity in constant conditions, were used. The animals were taken from a colony maintained in Haren that is based on individuals trapped in the Lauwersmeer (53°20'N:6°16'E). Animals were individually housed in lucite cages (25 × 25 × 30 cm) equipped with a running wheel and connected with a lucite nest box (17 × 11 × 13 cm) provided with wood shavings. Food (Hopefarms mouse pellets) and water were available *ad libitum*. Temperature (20 ± 1°C) and humidity (70%) were kept constant during the experiment. Animals were exposed to a 12:12 h LD cycle (lights on at 0600 h, 350 lux) for 2 weeks. After this, the animals stayed 4 weeks in constant low light conditions (LL: 2 ± 1 lux), while their wheel-running activity was recorded. Subsequently, they were exposed to a 2 week LD treatment (12:12 h, lights on at 0600 h, 350 lux). Seven voles were then killed at ZT 0 while the other seven were killed at ZT 6 under deep anesthesia with 0.75 ml 0.2% sodium pentobarbital. The brains were quickly removed from the skull and subjected to immersion fixation in 4% paraformaldehyde in 0.1 M phosphate-buffer for 6 h. The brains were rinsed with 0.01 M phosphate-buffer + 0.9% saline (PBS) and cryoprotected overnight in 30% buffered sucrose at 4°C. At the level of the hypothalamus, frontal sections (25 μ m) were cut with a cryostat. Free floating sections were rinsed with PBS and pre-incubated with normal goat serum (5%) and then incubated with a polyclonal IgG antibody rabbit anti-AVP (Truus '86; 1:4000) overnight at 4°C, or with a polyclonal rabbit IgG antibody raised against the catalytic subdomain (C20) of the PKC α isoform (Santa Cruz Biotechnology; 1:200) overnight at 4°C. After rinsing, the sections were exposed for 2 h at room temperature (RT) to biotinylated goat anti-rabbit IgG (F(ab')₂ fraction, (Zymed, 1:200). Subsequently, the sections were rinsed again in PBS and incubated with Streptavidin-Horseradish peroxidase (Zymed, 1:200) for 2 h at RT. The sections were thoroughly rinsed in PBS and Tris buffer (0.05 M, pH 7.4)

and visualized with diaminobenzidine (DAB; 30 mg in 100 ml Tris buffer, pH 7.4) as chromogen and 0.01% H₂O₂ for initiation of the staining reaction. Finally, sections were rinsed in PBS, mounted, air dried, and coverslipped for light microscopy and stereology.

Total numbers of AVP-ir and PKC α -ir neurons were estimated using the optical fractionator method described by West [21]. Cell counting was carried out using a transparent disector probe which was placed in the ocular of an Olympus CH40 light microscope. Cells were counted at 1000 × magnification. The fields of view were systematically sampled using a step size of 0.027 mm along the x-axis and 0.027 mm along the y-axis, and the disector counting frame was 729 μ m². The tissue (thickness) sampling fraction was 0.62. One fourth of all slices was used for AVP-staining and one fourth for PKC α -staining. Neurons were counted only when cytoplasmatic stainings were unambiguously apparent, and both hemispheres were sampled alternately. The minimum number of AVP-ir neurons and PKC α -ir neurons in one nucleus counted were 30 and 72, respectively. The numbers per nucleus were pooled for each animal and total numbers were calculated according to the above optical fractionator scheme.

A dense plexus of AVP-ir fibers and nerve terminals was seen in the dorsomedial part of the SCN at both ZT 0 and ZT 6. However, the situation at the level of AVP-ir cell somata changed drastically when animals were not killed at the immediate beginning of the light period but 6 h later. At ZT 6, a striking decrease in the number of AVP-ir neurons became apparent (Fig. 1A,B). Still some scattered AVP-ir neurons were seen throughout the SCN, but many more neurons in the dorsal portion of the SCN were devoid of cytoplasmatic AVP-staining. The mean number of AVP-ir neurons for the SCN at ZT 0 was 2194 (SEM 156) and 1270 (SEM 161) at ZT6. The difference between the mean numbers of AVP-ir neurons at the two ZT's was significant (Fig. 2, $P = 0.002$, Student's *t*-test, two tailed). Few scattered AVP-ir neurons were present just outside the SCN (Fig. 1B) at both ZT's.

At ZT 0 the SCN was delineated by numerous tightly packed PKC α -ir neurons (Fig. 1C), and no obvious difference was seen between dorsal, medial and ventral parts of the SCN in staining intensity and numerical density of PKC α -ir neurons. However at ZT 6, PKC α -immunoreactivity in the SCN differed considerably. The dorsolateral SCN showed a reduced PKC α -immunoreactivity, whereas in the ventromedial portion, close to the optic chiasm, a group of intensely stained PKC α -ir neurons was seen (Fig. 1D). The mean number of PKC α -ir neurons for the SCN at ZT 0 was 9897 (SEM 1030) and 6327 (SEM 826) at ZT 6. The difference between the mean numbers was significant (Fig. 2, $P = 0.005$, Student's *t*-test, two tailed). For each animal an AVP/PKC α ratio was calculated in order to examine if the decrease in the number of immunocytochemically stained neurons for both antigens was proportional. The average ratio did not differ significantly between ZT 0

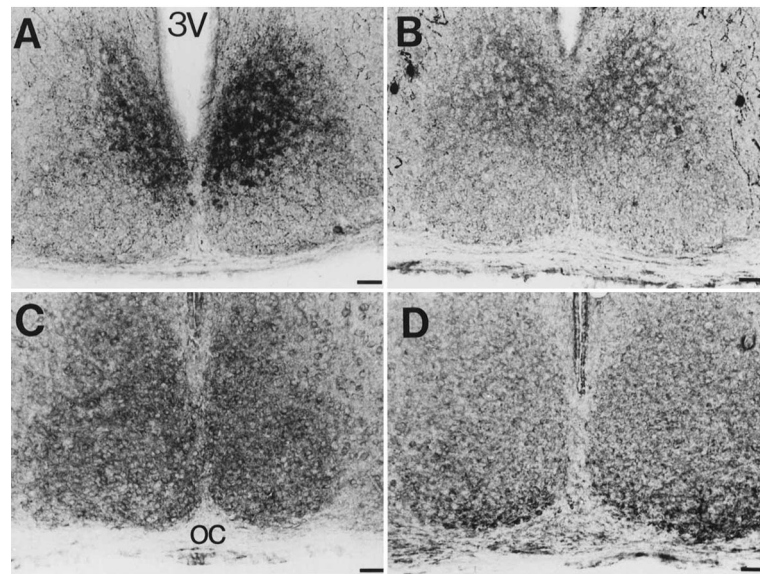


Fig. 1. Representative photomicrographs of the suprachiasmatic nucleus of two common voles (*Microtus arvalis*), entrained to a 12/12 h LD cycle, one sacrificed at zeitgeber time zero (A,C), and one at zeitgeber time six (B,D). The upper panel (A,B) shows AVP-immunoreactivity, while the lower panel (C,D) shows PKC α -immunoreactivity. 3V, third ventricle; OC, optic chiasm. Scale bars, 40 μ m.

(0.24 ± 0.034) and ZT 6 (0.23 ± 0.045 ; Student's *t*-test, two tailed, $P > 0.05$).

This study describes significant differences in the number of AVP and PKC α -ir neurons in the SCN at two distinct time points. Several studies addressed AVP localization and content in the rat SCN as well as release at different circadian times. Immunocytochemically, Okamura [13] showed a decrease in optical density of AVP in SCN neurons from ZT 2 to ZT 8. Micropunch studies [17,22] revealed an increase in AVP content in the early morning, which corroborates with AVP release patterns in rats [15] and voles (preliminary data on AVP release from vole SCN in vitro; Gerkema et al. in preparation). Circadian rhythmic voles show also a clear decrease in the number of AVP-ir SCN neurons between ZT 0 and ZT 6. We suggest that AVP-ir neurons release AVP either synaptically or non-synaptically at their varicosities during this 6 h time interval. The decrease in AVP-ir cell bodies is the consequence of transport of AVP out of the soma to the varicosities and the terminals. The reflection of plasticity in the AVP system in the vole SCN, which can be detected by immunocytochemistry, allows the study of the functional role of AVP within the circadian clock or its output system: AVP immunoreactivity in the SCN correlates strongly with variation of expression of circadian rhythmicity [6], and, as shown in this paper, changes with the circadian phase of the pacemaker. Moreover, loss of circadian rhythmicity correlates with dramatic reduction of AVP-ir neurons in the aging vole SCN [19].

PKC α labeling in the vole SCN differed from that in rat, mouse, rabbit and the diurnal Kusu rat, *Arvicanthis niloticus* [3,18]. In these species, PKC α -immunoreactivity was less uniformly distributed and found both in the dorsomedial and ventrolateral aspects of the SCN. However, PKC α observa-

tions were restricted to one time point in the early light period (ZT 2–3). It would therefore be of interest to determine whether comparison of different time points results in different patterns of PKC α -labeling in these species as observed here in voles.

Previously, it has been demonstrated that PKC mRNAs show circadian fluctuations [4]. Furthermore, McArthur et al. [10] found that PKCs can be activated at dusk and dawn by melatonin, which alters functioning of SCN neurons at ZT 23–0 and ZT 10–14. The function of PKCs within SCN neurons and their involvement in the intracellular signal transduction at clock level is currently under investigation. The decline in AVP-immunoreactivity in the SCN at ZT 6 as compared to ZT 0, and the concurrent decrease of PKC α -ir neurons dorsally supports the idea of direct coupling between AVP within the SCN and intracellular messengers linked to the V1a receptor. Stimulation of this receptor leads to intracellular PKC α activation, and it is known that acti-

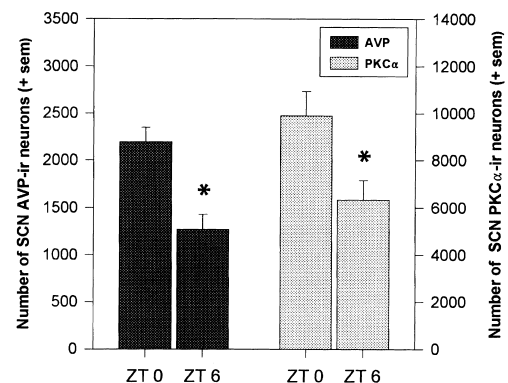


Fig. 2. Mean numbers of AVP-ir and PKC α -ir neurons in the vole SCN at zeitgeber time zero ($n = 7$) and six ($n = 7$). Scale bars represent SEM; *indicates significant decrease ($P < 0.01$).

vated PKC α (and PKC in general) is rapidly downregulated by proteases [14]. In turn this would lead to a reduction of PKC α -immunoreactivity as seen in the main portion of the SCN at ZT 6.

PKCs are also known to play an important role in the stabilization of long-term potentiation (LTP), a phenomenon of long-lasting enhancement of neuronal activity after repetitive synaptic stimulation. Circadian phase dependent LTP formation has been described in the SCN of rats after tetanic optic nerve stimulation [12], suggesting that LTP may be related to adaptation mechanisms to photic stimulation. Our data are consistent with this assumption. Perhaps PKCs and LTP are linked in the ventral part of the SCN, while the low density of AVP terminals in the ventral subregion suggests that, possibly, another (neuropeptide) system is involved in the activation of PKC α in the SCN. This idea is supported by the finding that neuropeptide Y can phase shift the clock in vitro via PKC [1]. Besides NPY, serotonin is also a likely candidate, based on the temporal sensitivity of the SCN with respect to serotonin and the spatial distribution of serotonin fibers in the SCN [9].

In summary, our results point towards a proportional turnover of AVP and its putative effector system PKC α , selectively in the SCN, depending on the time of day. These findings offer the possibility for further study of the dynamics of AVP-PKC α signal transduction in relation to the circadian clock function of the SCN. For example, AVP agonist and antagonist infusions into the SCN at differential ZTs could reveal the causal relation between AVP signal transduction and PKC α activation.

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